



# Using RFLP-PCR Technique in Determining Genotypes of *Giardia lamblia* from Diarrhea Cases in Children in AL-Diwaniyah City, Iraq

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## Abstract

**Introduction:** *Giardia lamblia* is one of the most prevalent intestinal protozoa in the world, which affect children in both undeveloped and developing countries. This study aimed to determine genotypes of the *Giardia lamblia* using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP)-PCR techniques. Additionally, the relationship between genotype patterns and their geographical distribution, gender, and age was investigated.

**Methods:** The current study included 926 samples of faeces of children suffering from diarrhoea, who visits the internal clinics at Teaching Hospital, and Child Hospital in AL- Diwanayah City from November 2012 - Jun 2013. For age groups of 1-12 years exclusively. The samples were examined using a direct mount wet smear, The positive samples were preserved without adding preservatives at a temperature of -20°C until the DNA extraction for *G. lamblia* genotyping by using PCR and RFLP-PCR technique.

**Results:** *Giardia lamblia* was detected in 2.15% (20/926) of samples from diarrhea cases in children by amplification of glutamate dehydrogenase gene (*gdh*) using two specific primers GDHiR and GDHiF. It was revealed that 7 samples belonged to genotype A (35%) and 13 samples belonged to genotype B (65%). All genotype A samples belonged to subgenotype All (100%), while genotype B samples belonged to subgenotypes BIII (53.61 %) and BIV (47.38 %). Genotype A was detected in children of 1-6 years of age while B genotype was detected in all age groups. Both of the genotypes have been detected in both genders (male and female) and genotype B was found in both urban and rural areas; however, its prevalence was higher in rural areas than in urban areas (100% and 30%, respectively).

**Conclusion:** There are two genotypes of *Giardia lamblia*, genotype A and genotype B, each of which has secondary genetic patterns which include All, BIII, and BIV.

**Keywords:** PCR-RFLP, Genotypes, *Giardia lamblia*, Diarrhea cases, Al-diwanayah, Iraq

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## Introduction

*Giardia lamblia* (*G. intestinalis*, or *G. duodenalis*) is one of the most prevalent intestinal protozoa in the world and one of the most common parasitic causes of Gastroenteritis, which primarily affect children in both undeveloped and developing countries. The infection rate ranges between 2% and 5% in industrial countries and it may exceed 30% in developing countries (1). This parasite causes giardiasis which is one of the most common causes of non-viral diarrhea among children which in turn leads to major health problems such as malabsorption and weight loss leading to delays in growth and development (2). Boontanom et al revealed that giardiasis infection is prevalent in all age groups, but young children are at the greatest risk for contracting giardiasis especially those attending childcare centers (nurseries) (3).

This parasite is spread throughout the world and it is one of the most common zoonotic agents in humans. Symptoms of *G. lamblia* infection appear in approximately 200 million people in developing countries located in Asia, Africa, and Latin America (4).

Diagnosis of *G. lamblia* infection depends on determining the parasite by microscopic examination such as wet mount smear or formalin-ether-acetate sedimentation technique and to enhance sensitivity, it is recommended that the test should be repeated several times, but it is often difficult to do it. Therefore, the amount of Antigen was determined by immunological tests such as enzyme-linked immunosorbent assay (ELISA) which has high sensitivity but it is very expensive (5).

Molecular methods such as Polymerase Chain reaction (PCR) and Real-time PCR (RT-PCR) are of high sensitivity



in identifying *Giardia lamblia* cysts in fecal samples, and the high sensitivity of these techniques has proven their superiority over microscopic examination in determining a parasite in feces, but unfortunately these methods have rarely been applied in undeveloped countries so far (6).

Modern molecular techniques such as real-time PCR or quantitative PCR (qPCR) are recently used in the diagnosis of certain genes belonging to *G. lamblia* such as Small subunit ribosomal RNA (ssrRNA), these techniques are valuable tools with higher sensitivity compared to conventional PCR in discriminating between strains and genotypes which can be used to understand the molecular epidemiology of *G. lamblia* (7).

Since there are many different genotypes of *G. lamblia* which can be distinguished on the basis of specialization to host, genetic mutations, or genomic mutations (8), many methods have been developed for use in molecular genetics. Moreover, in order to study the relationships between different genotypes isolated from their hosts, beta-giardin, glutamate dehydrogenase (*gdh*), and triosephosphate isomerase (*tpi*) genes can be used (9).

Several modern molecular methods have been developed to identify pathogens in the feces, such as the PCR. They are alternatives to traditional methods such as general stool examination. These methods are more sensitive than conventional microscopy. Restriction fragmentation length polymorphism (RFLP-PCR) is one of these techniques with more sensitivity and specificity to distinguish the genetic patterns of parasites, including *Giardia* spp. (10), using *gdh*, *tpi*, or beta-giardin genes (11).

The aims of this study were to determine genotypes of the *G. lamblia* that were found in the city of AL-Diwaniya using RFLP-PCR technique and to investigate the relationship between parasitic genotypes and their geographical distribution, gender, and age.

## Materials and Methods

### Collection of Stool Samples

The current study included 926 fecal samples of children suffering from diarrhea and complaining of abdominal pain who referred to Parasitology Laboratory at Diwaniya Teaching Hospital, Child Hospital, and some health centers in AL-Diwaniya city from November 1, 2012, to June 30, 2013. Children in the age groups of 1-12 were included in the study.

The samples were examined within a half-hour after collection by light microscope (Olympus -Japan) using direct wet mount smear and floatation method to detect the trophozoites and cysts stages of *Giardia lamblia*. The positive samples were stored without adding preservatives at the temperature of -20°C until the DNA extraction process was initiated for conventional PCR technique and RFLP-PCR technique in research laboratory, Department of Education and Zoonotic Diseases, Faculty of Veterinary Medicine.

### Direct Wet Mount Smear

The fecal samples were examined by direct wet mount smear to investigate the trophozoites or cysts stages of *Giardia lamblia* using a glass slide. A drop of 0.9% physiological saline or 1% Lugol's iodine solution was placed on the slide and mixed well with a small amount of feces with wooden chopsticks. Then, the slides were covered with the samples well and examined using a light microscope under 40X and 100X magnification (12).

### DNA Extraction

DNA was extracted from fecal samples using the Stool Genomic DNA extraction kit (Bioneer, Korea). The extraction was carried out according to the manufacturer's instructions and based on the method used in a previous study (13).

### DNA Profile

Purity and concentration (ng/μL) of DNA extracted from fecal samples were determined by reading the absorbance at a wavelength ranging from 260 to 280 nm in a Nanodrop spectrophotometer (Thermo, USA).

### Conventional PCR

#### Master Mix Preparation

The master mix for the conventional PCR was obtained using the Accupower® PCR Premix kit (Bioneer, Korea), consisting of 5 μL of DNA template, 1.5 μL of forward primer, and 1.5 μL of reverse primer, and 12 μL of water in a final volume 20 μL. After placing the mixture in the PCR tubes fitted with the kit, the tubes were carefully closed and mixed with the carburetor for 5 seconds, then it was transferred to the PCR thermocycler.

#### Thermal Cycling Program for Conventional PCR

The conditions used for DNA amplification were based on a previous study (14) which include initial denaturation at 94°C for 10 minutes, followed by 50 cycles of denaturation at 94°C for 35 seconds, 50 cycles of annealing at 61°C for 45 seconds, 50 cycles of extension at 72°C for 45 seconds and 50 cycles of final extension at 72°C for 7 minutes.

#### Agarose Gel Electrophoresis

The electrophoresis was performed in 1% agarose gel for reading the products of polymerase chain reaction (15).

#### RFLP-PCR Technique

Ten microliters of the PCR products at 432 bp were digested in 2 U of RsaI enzyme or 2 U of NlaIV in 1 X of enzyme buffer so that the final volume of the solution reached 25 μL. Then, it was autoclaved for 4 hours at 37°C to determine the genotype of the parasite.

RsaI Enzyme was used to differentiate between Subgenotypes BIII and BIV belonging to genotype B of *Giardia lamblia* based on the method used before (16).

NlaIv Enzyme was used to distinguish between subgenotypes AI and AII belonging to genotype A of *Giardia lamblia* (16). Then, the separation was done by electrophoresis in 2% agarose gel and ethidium bromide dye, and the products were read by UV spectrophotometry.

Determination of the Genotypes of *Giardia lamblia* Using the RFLP-PCR Technique Genotypes A, B and subgenotypes were determined depending on the method used by Leonhard et al (17) by comparing the obtained molecular size using two enzymes (RsaI and NlaIv) with the expected molecular size for each genotype, as shown in Table 1.

**Results**

**Results of *gdh* Gene Amplification Using the Conventional PCR**

A total of 926 fecal samples (2.15%) were examined using direct wet mount smear, among which 20 positive samples were microscopically identified. The amplification results were obtained successfully in all positive samples (20) of children with diarrhea (100%), and molecular size of *gdh* gene was determined at 432 bp, as shown in Figure 1.

**Results of PCR-RFLP Assay**

Results of the analysis of 20 samples of *Giardia lamblia*

**Table 1.** Genotypes of *G. lamblia* in Humans

Genotype Patterns	Used Enzyme	Expected Molecular Size (bp)
AII	NlaIv	20, 40, 50, 70, 80, 90, 120
BIII	NlaIv	50, 120, 290
BIII	RsaI	30, 50, 130, 300
BIV	RsaI	30, 50, 430

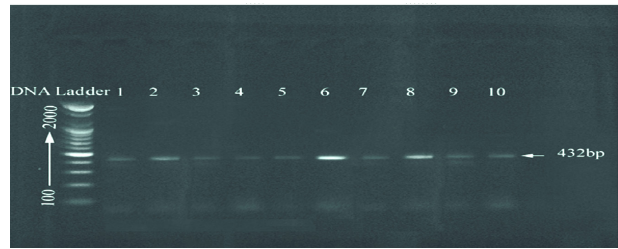
Source: Leonhard et al (15).

**Table 2.** Subgenotypes of *G. lamblia* Using RFLP-PCR

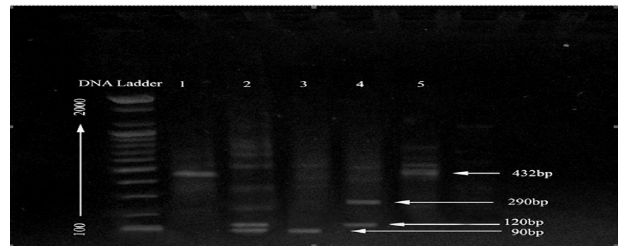
Parasite Genotypes	Used Enzymes	Expected Molecular Size (bp)	Obtained Molecular Size in the Study ( bp)
AII	NlaIv	20, 40, 50, 70, 80, 90, 120	90, 120
BIII	NlaIv	50, 120, 290	90, 120
BIII	RsaI	30, 50, 130, 300	130, 300
BIV	RsaI	30, 50, 430	430

**Table 3.** Distribution of Genotypes of *G. lamblia* Using RFLP- PCR

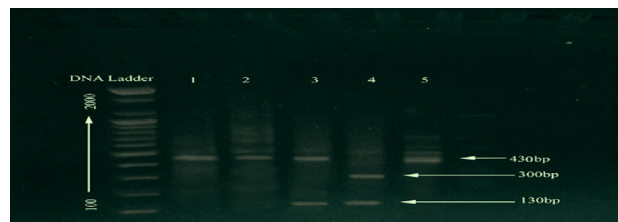
Genotype	Sample No.	Percent
AII	7	100
BIII	8	58.61
BIV	5	38.47
Total	20	100
AII	7	35
B	13	65



**Figure 1.** Electrophoresis of DNA Amplification of the *Giardia Lamblia* PCR Products on 1% Agarose Gel and Ethidium Bromide Dye for *gdh* Gene Using Specific Primers GDHiF and GDHiR. The columns 1-10 represent the PCR products of *gdh* gene at the molecular size of 432 bp, and L column represents the standard DNA ladder.



**Figure 2.** The *gdh* Gene Amplification on 2% Agarose Gel by PCR Using NLa IV Enzyme. Columns 1 and 5 represent the amplification of the gene at molecular size of 432 bp while column 2 and 3 represents the subgenotype AII of *Giardia lamblia* at molecular sizes of 90 bp and 120 bp, and column 4 represents subgenotype BIII at molecular sizes of 120 bp and 290 bp. The first column represents DNA Ladder.



**Figure 3.** The Amplification Product of *gdh* Gene on 2% Agarose Gel by PCR Using Rsa Enzyme I. columns 1, 2, 3, and 5 represent subgenotype BIV for *Giardia lamblia* at the molecular size of 430 bp while columns 3 and 4 represent subgenotype BIII for parasite which appears at molecular sizes of 130 bp (columns 3 and 4) and 300 bp (column 4), and L represents DNA Ladder.

using RFLP-PCR technique revealed that 7 (35%) samples belonged to genotype A and 13 (65%) belonged to genotype B, and the species belonging to the genotype B were divided into subgenotypes BIII (61.53%, 8 samples) and BIV (38.47%, 5 samples), while genotype A belonged to only subgenotype AII (100%, 7 samples), as shown in Tables 2 and 3 and Figures 2 and 3.

**Distribution of Genotypes of Giardia Lamblia by Gender Using RFLP-PCR**

The results of the current study showed that both of the genotypes (A and B) were detected in both genders, but

genotype B was more prevalent than subgenotype AII in males than in females, as shown in Table 4.

**Distribution of Genotypes of *Giardia lamblia* by the Age Group Using RFLP-PCR**

The current study investigated the genetic patterns of the parasite in different age groups and indicated that genotype AII had the highest percentage in the 1-3 age group (80%), while B genotype showed the highest percentage (100%) in the 6-12 age group, as shown in Table 5.

**Distribution of Genotypes of *Giardia lamblia* by Residence Area Using RFLP-PCR Technique**

The results of the study showed that the parasite genotypes were unevenly dispersed depending on the nature of the residence area. Genotype A was detected in 100% of the samples from

urban areas, while the genotype B and its different

subgenotypes were detected in both urban and rural areas, but they were more prevalent in rural areas (100%), as shown in Table 6.

**Discussion**

The results of the study showed a higher amplification rate using *gdh* gene in identifying the *Giardia lamblia* parasite compared to studies in which the same primers were used. In the current study, the positive percentage was 100%, which is higher compared to many previous studies. The *gdh* gene was detected in 100% of the people infected with the *Giardia* parasite (10). In another study, a prevalence of 52.9% was reported using the same gene (*gdh* gene) (18). In another study, the amplification of *tpi* gene reached 94% (11), while it was 55% in another study (19) using formalin or potassium chromate. In another study, a percentage of 98.1% was reported as well. Previous studies revealed the possibility of DNA replication in some obtained samples, which may be due to the lack of some PCR materials such as bile salts, haemoglobin, saccharides, or fats resulting from mucus and bacteria that may affect the results (20-22).

Results of molecular identification of *Giardia lamblia* using RFLP-PCR technique, which is a very sensitive technique for determining genetic variations and distinguishing between subgenotypes, showed the dominance of genotype B with a prevalence of 65% compared with genotype A that reached 35% in Diwaniyah city, this is consistent with a survey (23) conducted in Iran using *gdh* gene. The highest percentage of infections with genotype B was 66.66% compared with genotype A which amounted to 33.33%, which is consistent with a previous study (13) in which genotype B (61%) was more prevalent than genotype A (39.1%) using the *tpi* gene.

Results of molecular identification of *Giardia lamblia* have indicated different subgenotypes of parasite in Diwaniya city. Genotype B samples belonged to subgenotypes BIII (53.61%, 8 samples) and BIV (47.38%, 5 samples), while genotype A samples belonged to subgenotype AII (100%, 7 samples), which is consistent with a previous study (24).

The results of the current study are consistent with the results of many previous studies (16,25) which indicated the presence of multiple genotypes (genetic patterns) of *G. lamblia* in human and other hosts which include A, B, C, D, E, F, and G. Genotypes A and B are found only in human (11). Therefore, RFLP-PCR technique was used as a very sensitive method to determine genetic variations of the parasite and distinguish between subgenotypes by molecular techniques using *tpi*, *fc4*, and *gdh* genes, which is consistent with the previous studies (26,27), where they proved that it has the ability to distinguish between genotypes A and B, identify differences between them and to distinguish between the subgenotypes A and B.

**Distribution of Genetic Patterns by Gender, Age, and Residence Area Using RFLP-PCR**

The results of the current study showed that all the children in different age groups and both genders are exposed to the infection with both genotypes of *G. lamblia* (A and B);

**Table 4.** Distribution of Genotypes of *Giardia lamblia* by Gender

Gender	Number of Positive Samples	Genotype	Percentage of Genotype (%)
Male	10	AII	3 (30)
		B	7 (30)
Female	10	AII	4 (40)
		B	6 (40)
Total	20	AII	7 (35)
		B	13 (65)

**Table 5.** Distribution of Genotypes of *Giardia lamblia* by Age

Age Groups	No. of Positive Samples	Genotype	Percent
<3	5	AII	4 (80)
		B	1 (20)
>3-6	5	AII	3 (60)
		B	2 (40)
>6-9	5	AII	-
		B	5 (100)
>9-12	5	AII	-
		B	5 (100)
Total	5	AII	7 (35)
		B	13 (65)

**Table 6.** Distribution of Genotypes of *Giardia lamblia* by the Residential Area

Residence Area	No. of Positive Samples	Genotype	Percent
Urban	10	AII	7 (70)
		B	3 (30)
Rural	10	AII	-
		B	10 (100)
Total	20	AII	7 (35)
		B	13 (65)



however, it was revealed that subgenotype AII is capable of infecting the children in the age group 1-5 years, while subgenotype B is able to infect all age groups of children (1-12 years), and that may be due to the nature of immune system and parasite virulence factors, these results are in line with previous studies (25,26,28).

Additionally, the current study showed the presence of the *G. lamblia* in all 20 diarrheal samples of the children (100%) examined, and these results were similar to those recorded in a previous study in which a close relationship was found between parasitic infection and diarrhea cases in children infected with genotype A of the parasite (29). However, in another study no close relationship was found between changes in the digestive system and genotypes of the *G. lamblia* in confirmed cases (30). Moreover, in another study it was revealed that genotype B of *Giardia lamblia* is responsible for most cases of diarrhea in England using *gdh* gene (13). These differences in the results may be due to changes in the nature of absorption of nutrients caused by the *G. lamblia*, which affects the absorption of fat in the body causing fatty diarrhea (11).

### Conclusion

PCR-RFLP technique used in this study was able to identify and distinguish between all the different genotypes of *G. lamblia* in the study area.

### Conflict of Interests

The authors declared that no competing interests exist.

### Ethical Issues

In this research, ethical considerations have been fully observed.

### Acknowledgement

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### Authors' Contribution

We did writing, editing of the manuscript, statistical analysis and data collection.

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